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Transport of Viruses Through Fetal Membranes: An In Vitro Model of Perinatal Transmission

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A model system for perinatal transmission of viral infections was developed and transport of infectious virus particles through fetal membranes was investigated. Viruses of different families known to cause serious intrauterine infections were selected, including relevant and model viruses: the DNA-viruses HSV-1 and -2 as well as the animal herpes viruses BHV-1 and SHV-1, the RNA-virus BVDV as a model for hepatitis C virus, HIV-1 and -2, and PPV as a model for parvovirus B19. Migration of infectious virus from the maternal to the fetal side of the membrane could be detected as early as 20 min after the start of incubation. A peak of virus migration was observed after 1–2 hr. 0.02–1% of HSV-1 and 0.03–0.2% of HSV-2 were transported from the maternal side of the membrane to the fetal side. Only 0.01% of PPV migrated to the fetal side, whereas no transport of BVDV was observed. HIV-1 (1.4%) and HIV-2 (0.8%) seemed to be transported at higher rates. The concept of an active transport of infectious virus is compatible with the kinetics of penetration of the fetal membrane. The question of whether different receptors for the individual viruses on the cellular surface account for differences in virus transport will require further investigation. The fetal membrane acts as a protective barrier for the fetus, reducing greatly infectious titers or even preventing completely penetration of virus. ***J. Med. Virol. 54:313–319, 1998.***

INTRODUCTION

Transmission of viruses from mother to child accounts for serious diseases in infants. Vertical transmission of the human immunodeficiency virus (HIV) presents an increasing problem, especially in developing countries. According to WHO, about 2.6 million children have been infected with HIV since the start of the global epidemic, 1.4 million of whom have died (WHO, 1997). The rate of mother-to-child transmission of HIV-1 varies from 13–42% in Europe [The European Collaborative Study, 1991, 1992, 1994, 1996; Simonon et al., 1994] to twice as high in Africa [Dabis et al., 1993].

Perinatal infection with herpes simplex virus (HSV) may lead to damage to the central nervous system. Parvoviruses may be transmitted in utero [Cohen, 1995; Mengeling, 1975] mainly during the first and second trimester. Fetal death occurs in about 10% of pregnancies complicated by parvovirus B19 infections, [Brown and Ritchie, 1985]; however, there is no evidence of permanent damage to the fetus who survives infection and is born alive. Infection of the fetus with hepatitis B (HBV) or hepatitis C virus (HCV) can cause a carrier state with persistent virus production and carries an increased risk of virus dissemination and a high risk of developing liver cirrhosis and malignancies. Vertical transmission of HCV is a rare event; only mothers with high titers of circulating virus during pregnancy or simultaneous HIV infection infected the fetus [Ohto et al., 1994; Zanetti et al., 1995].

Transmission of viruses may occur in utero through the placenta, or postnatally via breast feeding. Perinatal transmission may be due to contact of the infant's skin and mucosa with maternal blood and vaginal secretion during delivery. About 50% of HIV transmissions are estimated to occur close to the time of delivery [Bryson et al., 1992; Mofenson, 1995]. Several models for transplacental transmission of HIV [Curtis et al., 1991] and HSV [Norskov-Lauritsen et al., 1992] have been established in recent years. The transmission of HIV by infection of trophoblasts and villous Hofbauer cells, the placental macrophages, has also been investigated [Kesson et al., 1993].

Like the placenta, the fetal membranes are part of the maternal-fetal interface. The inner membrane (amnion) consists of a single layer of cuboidal epithelial cells firmly attached to a distinct basement membrane connected to avascular connective tissue. The outermost layer of the amnion is contiguous with the second membrane (chorion laeve) which also consists of connective tissue vascularized by fetal blood vessels and a layer of dotted cytotrophoblasts within. The outermost layer of the chorion is slightly attached to the maternally derived decidua. During labor and delivery the chorion is detached from the decidua; however, clusters

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of decidual cells remain adherent to the chorion after delivery of placenta and membranes.

Migration of peripheral blood mononuclear cells through the amnion membrane has been demonstrated by electron microscopy in a model system simulating inflammatory processes by chemotactic stimuli [Bakowski and Tschesche, 1992]. Peripheral blood cells infected with viruses may penetrate the fetal membranes and participate in vertical transmission events. However, infection of the fetus by cell-free virus during viremia in vivo cannot be excluded. An experimental model was designed to study the penetration of cell-free virus through the fetal membranes.

MATERIALS AND METHODS

Preparation of Virus Stock

Human herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) [Ejercito et al., 1968] were propagated in Vero cells, swine herpes virus type 1 (SHV-1) [Bartha et al., 1969] in mink lung cells (ML), bovine herpes virus type 1 (BHV-1) (vaccination strain Bocine®, Cutter Animal Health, Shawnee, Kansas) in Georgia bovine kidney cells (GBK), porcine parvovirus (PPV) [Mayr et al., 1968] in swine testis cells (ST), bovine virus diarrhea virus (BVDV) as model virus for hepatitis C [Nowak et al., 1992] in calf lung cells (KL). KL and ST cells were propagated in MEM supplemented with 5% fetal calf serum, Vero, GBK, and ML cells in D-MEM with 10% fetal calf serum, and the lymphoid suspension cell Molt 4 clone 8 cells (Molt 4/8) in RPMI1640 with 10% fetal calf serum. All media and fetal calf serum (heat inactivated for 30 min at 56°C) were supplied by Gibco.

Subconfluent cell monolayers in tissue culture flasks or tissue culture plates (Nunc) were infected with the respective virus at a multiplicity of infection of 10^{-3}. Supernatants were harvested when a prominent cytopathic effect (CPE) was visible (1–6 days post infection; dpi), cell debris were removed by centrifugation (10 min, 3000 rpm, 4°C), and the cell-free supernatant was stored in aliquots at −70°C.

HIV-1 (O) [Gürtler et al., 1993] and HIV-2 strain SBL 6669 [Albert et al., 1987] were propagated in Molt 4/8 cells. One week after infection cells were mixed with uninfected cells (1:1) and cocultivated for 2–3 days until a prominent CPE was observed. Cell-free virus was prepared as described above.

Titration of Virus

Virus was titrated by standard microtitration assay. Cells (100 μl/well) were seeded into 96-well microtiter plates (Nunc) at a concentration of 2×10^5/ml for monolayer cells. Virus samples were diluted serially threefold, 8 wells were inoculated with 100 μl aliquots of the respective virus dilution and incubated at 37°C. Adherent cells were washed twice gently with phosphate buffered saline (PBS) after 2 hr of absorption. At the time when the virus-specific CPE on monolayer cells was visible (1–7 dpi after inoculation), cells were fixed with 4% formaldehyde, stained with Giemsa solution (Merck, Darmstadt), and evaluated under an inverted light microscope. Titration of HIV was carried out in duplicate in 24-well tissue culture plates (Nunc) on Molt 4/8 cells (2×10^4/ml). The strains HIV-1 (O) and HIV-2 SBL H6669 were used because of good syncytia formation. Cytotoxicity of cell culture supernatants (see above) necessitated predilution by a factor of 1:100. The titration was evaluated 15–20 dpi, medium was replaced twice a week, and cells were diluted when required. The tissue culture infectious dosis $TCID_{50}$/ml was determined by the method of Reed and Münch [1938].

Fetal Membranes

Fetal membranes were obtained immediately after normal deliveries. The fetal parts of the membranes (chorion and amnion) were removed from the placenta, washed three times in sterile phosphate buffered saline (PBS), and transferred to cell culture medium. A 5 × 5 cm square was cut from the membrane, positioned on a plastic ring (diameter 22 mm, height 12 mm), and fixed with a rubber ring and titanium clips forming a tight chamber. This chamber was placed on a filter membrane in 6-well cell culture plates (Nunc). The fetal membrane divided the cell culture vessel into two compartments, the maternal side (chorion) facing the upper compartment and the fetal side (amnion) the lower compartment.

One ml of the appropriate cell-free virus stock was transferred into the upper compartment and 3 ml culture medium into the lower compartment. After incubation at 37°C, the culture medium in the lower compartment was removed at indicated times and stored in 1 ml aliquots at −70°C for virus titration. HIV was titrated immediately. Fresh medium was added to the lower compartment. At least two independent experiments with independently prepared virus stocks were carried out for each virus.

RESULTS

Most viruses investigated penetrated the fetal membranes, although great variation in the amount of transported virus was observed (Table I).

HSV-1 and *-2* passed the fetal membrane with a peak of virus transport after 1–2 h incubation. However, virus could be detected in the lower compartment as early as 20 min after transfer of virus to the upper compartment. In general, less than 1% of the infectious virus in the maternal compartment migrated to the fetal side during the 24 hr observation time. The amount of virus transported varied with individual membrane donors, as shown for HSV-1 (Table II). Virus migration during 24 hr ranged from 0.02–1.0% (HSV-1) and 0.03–0.2% (HSV-2). Minor variation in the amount of virus below the fetal membrane was observed when using different membrane patches of the same donor (Fig. 1). Degradation of the fetal membrane did not cause the decrease of virus transport after longer incubation periods, since incubation of the membrane in culture medium prior to transfer of virus into

TABLE I. Transport of Infectious Virus Through Fetal Membranes ($TCID_{50}$/ml)

	T_0[a]	20 Min[b]	40 Min[b]	1 Hr[b]	2 Hr[b]	4 Hr[b]	6 Hr[b]	24 Hr[b]	% T_0[c]
HSV-1	3.7×10^6	1.3×10^3	1.1×10^4	2.9×10^4	2.4×10^3	1.8×10^3	52	2.5	0.5
HSV-2	6.0×10^5	12	69	2.1×10^2	71	27	40	1	0.05
BHV-1	2.2×10^7	nd	nd	5.4×10^2	7.2×10^2	7×10^3	2.6×10^2	58	0.07
SHV-1	1.1×10^7	nd	nd	3.5×10^2	7.2×10^2	2.1×10^3	5.5×10^2	2.5×10^2	0.06
PPV	1.3×10^7	nd	nd	2.7×10^2	4.7×10^2	2.1×10^2	23	35	0.01
BVDV	3.2×10^6	nd	nd	0	0	0	0	0	0
HIV-1	8.4×10^4	nd	nd	1.8×10^2	3.1×10^2	1.8×10^2	1.8×10^2	60	1.4
HIV-2	3.5×10^5	1×10^2	35	1×10^2	20	35	nd	nd	0.2

One representative experiment is shown for each virus.
[a]Titer at time 0 in maternal compartment.
[b]Titer in fetal compartment.
[c]Total infectious virus in fetal compartment (% of T_0) at the end of the experiment.
nd = not done.

TABLE II. Transport of Infectious HSV-1 Per Hr ($TCID_{50}$/ml) Through Fetal Membranes From 5 Different Donors (FM 1–5)

	T_0[a]	20 Min	40 Min	1 Hr	2 Hr	4 Hr	6 Hr	24 Hr	% T_0[c]
FM 1	3.7×10^6	1.3×10^3	1.1×10^4	2.9×10^4	2.4×10^3	1.8×10^3	52	2.5	0.5
FM 2	3.7×10^6	2×10^3	8.1×10^3	2.9×10^4	9.6×10^3	4.8×10^3	8×10^2	1×10^2	1.0
FM 3	9.2×10^6	1.4×10^3	1.1×10^3	4.8×10^2	5×10^3	1.5×10^2	50	0	0.07
FM 4	1.9×10^7	nd	nd	3.3×10^4	6.7×10^3	4.0×10^2	10	7	0.2
FM 5	6.8×10^7	nd	nd	2.6×10^3	9.3×10^3	4.7×10^2	15	4	0.02

See legend to Table 1.

the maternal compartment did not influence transport kinetics of HSV-1. Cumulative virus titer ($TCID_{50}$/ml) in fetal compartment at the times indicated: without preincubation of fetal membrane 2.8×10^4 at 40 min, 7.0×10^4 at 2 hr, 1.4×10^5 at 24 hr; 2 hr preincubation at 37°C 2.8×10^4 at 40 min, 9.5×10^4 at 2 hr, 1.8×10^5 at 24 hr; 4 hr preincubation at 37°C 1.3×10^4 at 40 min, 6.3×10^4 at 2 hr, 1.1×10^5 at 24 hr. A prolonged time interval between delivery and the preparation of the amniochorion, while the fetal membrane was kept in phosphate buffered saline, did not alter the kinetics of virus transport either. Cumulative virus titer ($TCID_{50}$/ml) in fetal compartment: preparation of fetal membrane 0 hr after delivery 1.6×10^5 at 40 min, 3.7×10^5 at 2 hr, 4.8×10^5 at 4 hr; 2 hr after delivery 40 min 6.5×10^4, 2 hr 1.0×10^5, 4 hr 1.7×10^5, 4 hr after delivery 40 min 4.7×10^4, 2 hr 7.6×10^4, 4 hr 9.8×10^4. The amount of virus transported was to some degree dependent on the titer in the maternal compartment as shown for HSV-1 (Fig. 2). A 10-fold dilution of virus on the maternal side resulted in an equivalent decrease of virus on the fetal side. However, a 100-fold dilution resulted in only a slight further decrease of virus titers in the fetal compartment. The transport of HSV-1 and -2 was not directional; transport from the maternal to the fetal side occurred at approximately the same rate as from the fetal to the maternal side (amnion side facing the upper compartment and chorion side facing the lower compartment, data not shown).

BHV-1 and *SHV-1* crossed the fetal membrane at rates similar to HSV-1 and -2 (Table I), demonstrating that transport of herpes viruses seems to be not species specific.

The transport kinetics for the small unenveloped *PPV* was comparable to the large enveloped herpes viruses with maximum transport during the first 2 hr of incubation; however, only about 0.01% of infectious virus migrated through the fetal membrane.

BVDV, a suitable model for HCV, did not cross the fetal membranes. Two independent virus preparations were investigated. Inactivation of BVDV by neutralizing antibodies in the fetal calf serum were excluded.

HIV-1 and *-2* migrated through the fetal membranes with approximately the same transport kinetics as HSV-1 and -2 (Fig. 3), but HIV-1 seemed to be transported more efficiently than HIV-2 (1.4% vs. 0.2%).

Passive diffusion through the fetal membrane was investigated using crystal violet as indicator molecule, the dye concentration in the upper compartment being 0.5 mMol/l. Diffusion of the dye through the membrane was determined by measuring the extinction at 620 nm in the lower compartment. Crystal violet was not detected below the membrane during the first 2 hr, whereas 0.5% of the dye had diffused after 4 hr, 1% after 6 hr, and 3.6% after 24 hr. Diffusion of the small dye molecule (MW 408 Dalton) was not detectable initially and was slow thereafter, indicating that the membrane was mechanically intact.

Thermal inactivation of virus during the incubation period did not account for the decreasing transport rates through the membrane at later time intervals. HSV-1 was stable at 37°C for 4 hr, infectivity decreased to 58% of starting titer after 6 hr and to 3% after 24 hr. HSV-2 titers remained at 100% for 24 hr. BHV-1 and SHV-1 titers fell to 75% of the starting titer after 6 hr and to 30% or 15%, respectively, after 24 hr at 37°C. PPV titer decreased to 76% after 24 hr and BVDV titers to 73% after 4 hr and to 11% after 6 hr and 24 hr. Only HIV was inactivated substantially by incubation at 37°C: Infectious HIV-1 decreased after 2 hr incubation

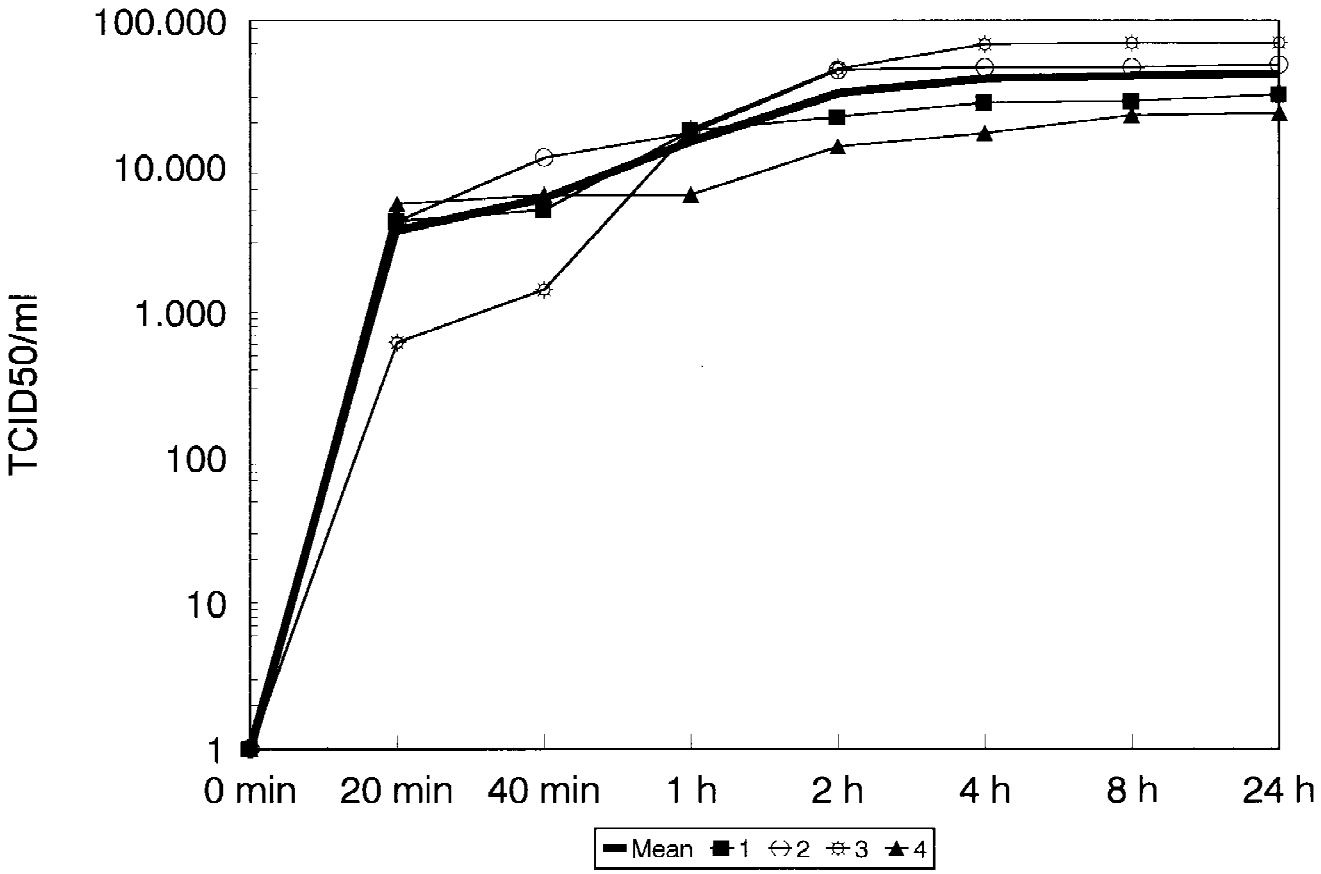

Fig. 1. Transport of HSV-1 through adjacent areas of a fetal membrane. Shown are the cumulative infectious titers ($TCID_{50}$/ml) detected at different time points in the fetal compartment.

to 11%, and after 24 hr to 2% of the starting titer, whereas HIV-2 was more stable initially with 50% residual infectivity at 4 hr and 0.2% at 24 hr.

Due to varying rates of virus transport through fetal membranes derived from different donors and, to a lesser degree, of different areas of the same membrane, the amount of virus transported cannot be compared directly. For example, about 1% of HIV-1 penetrated the fetal membrane despite comparatively low titers of virus stocks and a pronounced thermal instability which significantly reduced titers during the course of the experiment. Up to 1% of HSV-1, with higher starting titers and better thermal stability, but only 0.01% of PPV, with a starting titer more than 100 times higher and high thermal stability, were transported to the fetal side.

DISCUSSION

Penetration of cell-free viruses through the amniochorion was investigated using the model system for vertical transmission of viral infections. The viruses investigated included the relevant enveloped viruses HSV-1 and -2 and the human retroviruses (HIV-1 and -2), the enveloped model viruses (BHV-1, SHV-1 and BVDV) as well as the unenveloped parvovirus (PPV). It could be shown that viruses belonging to different virus families were transported through the membrane layers; penetration was rapid as infectious virus could be demonstrated as early as 20 min after adding virus at the opposite side of the membrane. The amount of transported virus reached a peak after 1–2 hr incubation and decreased later on, with little virus penetrating the membrane at 24 hr. These kinetics of virus migration cannot be accounted for by a passive diffusion process through intercellular spaces in the membrane. Diffusion of the small dye molecule crystal violet occurred at a much slower rate, and the dye could be detected below the membrane only after several hours incubation. Loss of viability of the amniochorion was excluded as a reason for decreasing transport activity, since prior incubation of the membrane in medium for up to 4 hr did not alter the kinetics of HSV-1 transport. Thermal inactivation of the virus in the maternal compartment did not account for the diminished migration to the fetal compartment either, as for example the herpes viruses HSV-1/-2, BHV-1, and SHV-1 were stable at 37°C for up to 24 hr.

Virus penetration through the fetal membrane may involve transcytosis of internalized virus particles. Immunoglobulin A-mediated transcytosis through polarized cell monolayers via the immunoglobulin receptor has been described for Epstein–Barr virus without evi-

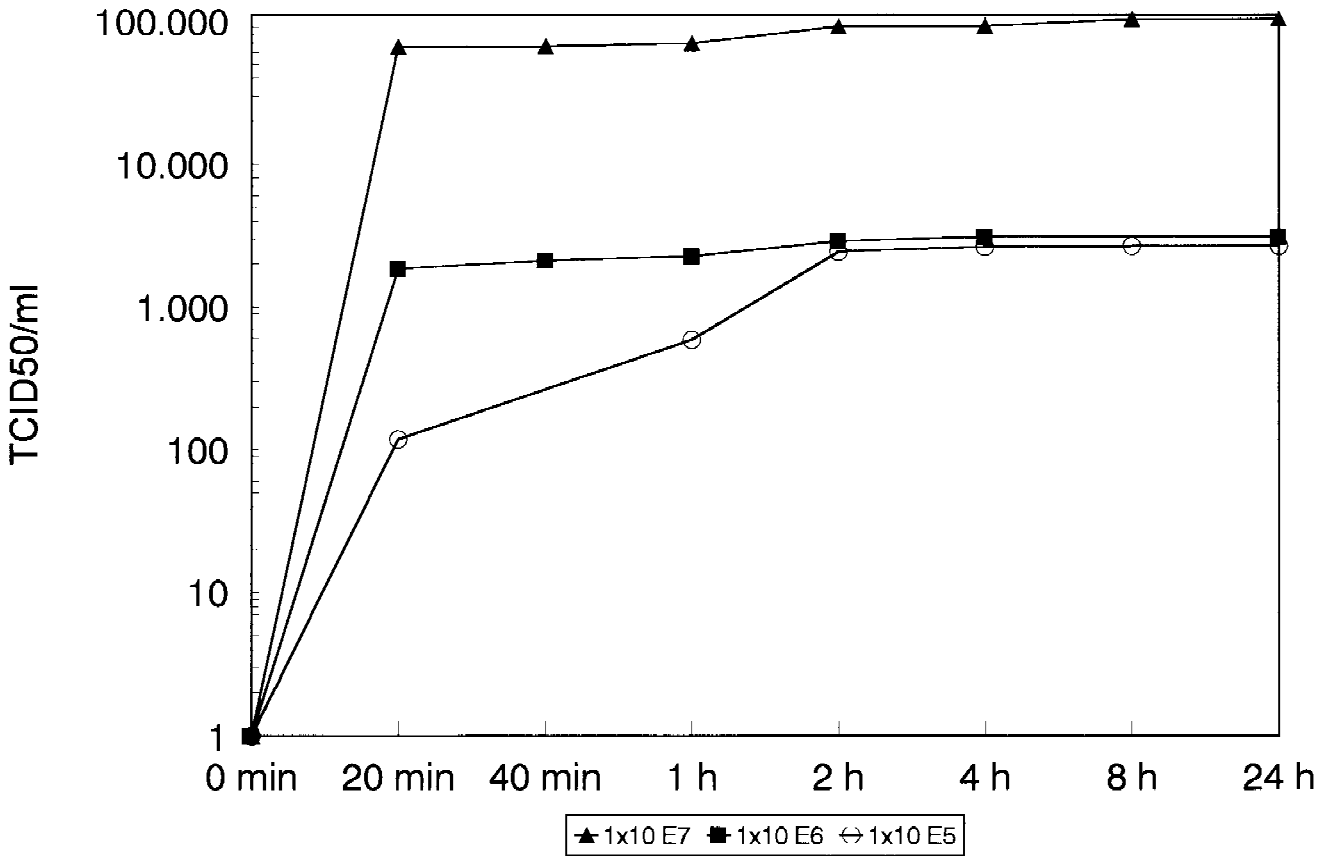

Fig. 2. Influence of the amount of HSV-1 in the maternal compartment on viral transport through the fetal membrane. Virus stock was diluted 1:10, 1:100 and 1:1000. Shown are the cumulative infectious titers ($TCID_{50}$/ml) detected at different time points in the fetal compartment.

dence of infection [Gan et al., 1997]. Receptor-mediated transcytosis has been proposed for the transport of HIV through epithelial monolayers, too. However, transcytosis was observed only upon cell–cell contact of HIV-infected cells with an epithelial cell monolayer, and cell-free HIV did not cross the epithelial barrier [Bomsel, 1997]. In our ex vivo model of vertical transmission through a complex biological membrane comprising several layers, transport of cell-free virus was demonstrated in a titer-dependent manner. The kinetics of virus transport were compatible with an active transport mechanism of infectious virus, probably using different cell surface receptors for individual viruses. At least part of the putative receptors do not seem to be species specific, as the animal viruses BHV-1 and SHV-1 penetrated the human fetal membrane at rates comparable to the human herpes viruses. BVDV is not transported through the fetal membrane, supporting the hypothesis of an active transport mechanism for the other viruses and excluding diffusion through labor-induced tears or the intercellular space in the loose structure of the membrane.

The results are in agreement with epidemiological and clinical data on the transmission of viruses from mother to child. The risk of HSV transmission from a maternal genital HSV infection can be reduced by cesarean section, especially after rupture of the membranes [Stone et al., 1989]. Primary infection is associated with high virus titers in the genital tract [Corey and Spear, 1988]. A correlation of the amount of virus crossing the membrane and the infectious titer on the maternal side could be demonstrated for HSV-1 in the model system. Only up to 1% of infectious virus penetrated the fetal membrane under the experimental conditions.

HIV is transmitted in Europe and North America by 15–20% of HIV-positive mothers to the child. P24 antigen has been detected in amniotic fluid of seropositive women [Viscarello et al., 1992], providing evidence for in vivo penetration of HIV through the fetal membrane. The risk of vertical transmission of HIV was increased when invasive procedures compromising the integrity of the fetal membrane, e.g. amniocentesis and amnioscopy, had been used [Mandelbrot et al., 1996]. Among HIV-infected mothers who gave birth later than 4 hr after the rupture of fetal membranes, the rate of transmission of HIV-1 to infants was 25% compared to 14% among mothers who delivered earlier [Landesman et al., 1996]. In our in vitro model approximately 1% of infectious HIV penetrated the fetal membrane, showing that the intact amniochorion could significantly reduce the amount of infectious HIV on the fetal side.

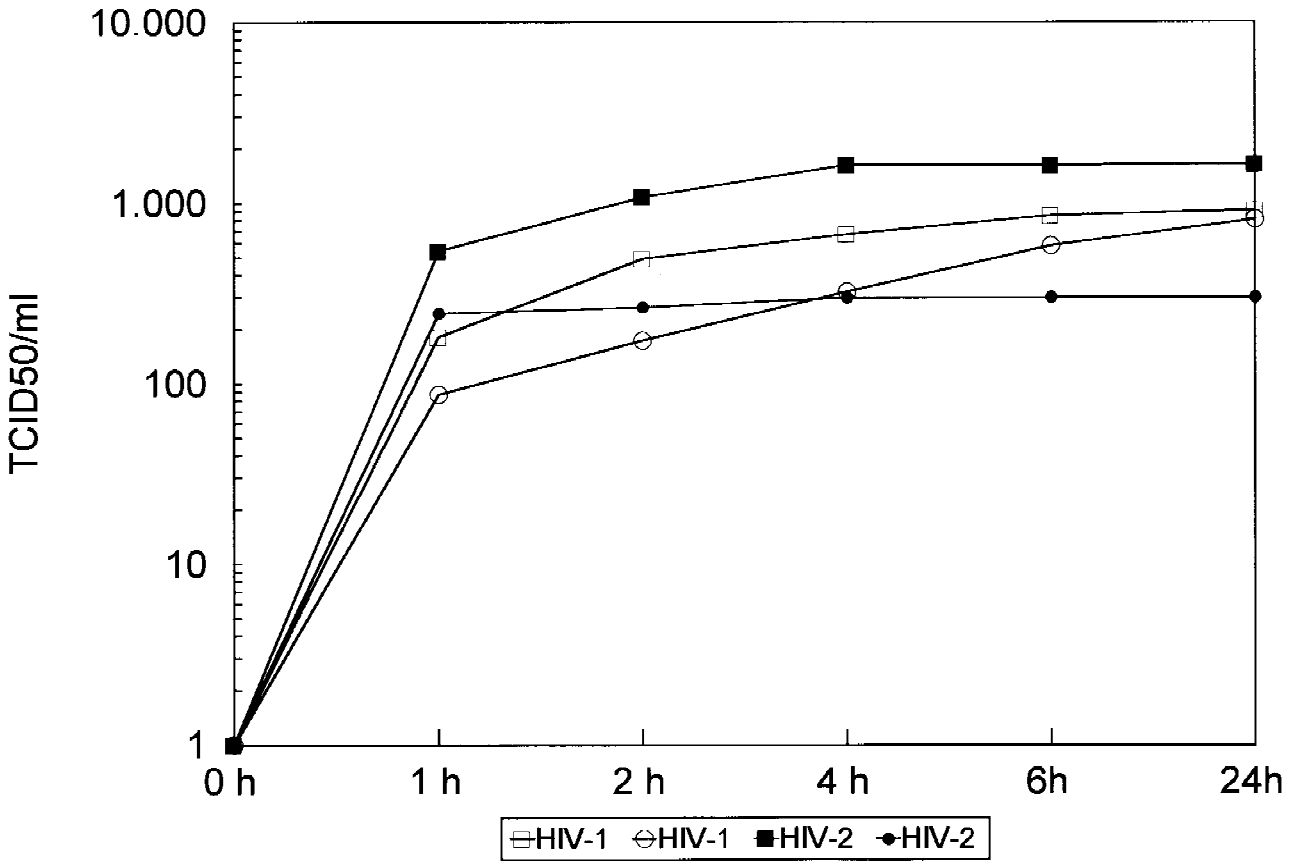

Fig. 3. Transport of HIV-1 and -2 through the fetal membrane. Shown are the cumulative infectious titers ($TCID_{50}$/ml) detected at different time points in the fetal compartment.

Simian immunodeficiency virus (SIV) infection of neonatal rhesus monkeys occurred at a high rate (6 out of 7 animals) when SIV was injected into the amniotic fluid late in gestation [Fazely et al., 1993]. These results demonstrate that perinatal transmission becomes a frequent event when viral load in the amniotic fluid is high.

Transfer of HIV-1 p24 antigen through the placenta to the fetal circulation has been demonstrated [Bawdon et al., 1995], and a perfusion model suitable for the investigation of transfer of infectious virus through the placenta has been developed [Polliotti et al., 1996], but data to estimate the significance of infection via the fetal membrane compared to the transplacental route is not yet available.

The in vitro model of perinatal virus transmission shows that the fetal membrane might act as protective barrier for the fetus, greatly reducing infectious virus titers on the fetal side or even completely preventing penetration of virus.

The in vitro system will be used to identify the putative cellular receptors involved in transcytosis of different viruses through the fetal membrane. The identification of receptors, presumably distinct for different viruses, is prerequisite to search for inhibitors which might be able to reduce the rate of vertical virus transmission due to transcytosis of cell-free virus.

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